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EFFECTS OF RADIOFREQUENCY FIELDS ON EXCITABLE TISSUES

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OCCUPATIONAL AND ENVIRONMENTAL HEALTH DIRECTORATE
Brooks Air Force Base, TX 78235-5000

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NOTICES

This report has been reviewed and is approved for publication.

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13. ABSTRACT *(Maximum 200 words)*

A millimeter wave exposure device with an attached fluorescent microscope was built. The fluorescent electrical potential-sensitive dye merocyanine 540 demonstrated the best response in the millimeter radiation field. Analysis of data from experiments in which miniature end-plate potentials of neuromuscular preparation were measured before, during, and after exposure to 51.72 and 51.81 GHz at 5 mW/cm^2 yielded no significant differences in rise time, decay time, duration, or amplitude of the potentials. These measurements were made with high-resistance electrodes.

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EFFECTS OF RADIOFREQUENCY FIELDS ON EXCITABLE TISSUES

OBJECTIVES

The long-term objective of this project was to determine the feasibility of studying the effects of radiofrequency radiation (RFR) at millimeter wavelengths on excitable tissues.

To accomplish this:

A thorough review of the biological literature and data concerning the nonperturbing measurements of membrane potentials was carried out.

Measurement approaches most adaptable to RFR fields were determined.

A prototype system, using fluorescent probes and microelectrodes, was designed and developed.

The feasibility of simultaneously examining optically and electrically, well-defined, in vitro, fresh neuromuscular preparations, as well as cultured muscle cells and fibers, was determined.

Preliminary experiments at millimeter wave frequencies and low-power densities were carried out to validate the feasibility of monitoring resting potentials and miniature end-plate potentials (MEPPs).

INSTRUMENTATION AND SYSTEM DESIGN

The prototype system was designed and built to enable simultaneous exposure to millimeter waves, a fluorescence excitation beam, and microelectrode stimulation. Resting potentials and MEPPs were recorded before, during, and after RFR exposure by this on-line system.

The microwave system which was interfaced with the experimental system, included millimeter waveguide components in the 26-75 GHz range, a backward wave oscillator (BWO), attenuator, frequency meter, thermistors, and power meters, for measuring incident and reflected power, as well as radiofrequency (RF) switches and microwave horns (Figs. 1 and 2). Experimental capability included both continuous wave and modulation.

A Perkin-Elmer spectrophotometer was changed to enable the interfacing of optical fibers with excitation and emission optics for transmission of fluorescence signals to and from the specimen (Figs. 3a and 3b). A steel plate, mounted on rubber

balls and a tube for stabilization, was insulated from a brass plate containing water channels for temperature control (Figs. 2 and 4). The sample chamber with sapphire plates (Fig. 5) rests on the brass plate in apposition to the microwave horn (Fig. 4). A microscope with a retractable arm enables field visibility. A black box designed to cover the sample chamber so that fluorescent monitoring would be enhanced was replaced by a blackened Faraday cage encased in Metglass, on a sand loaded table. It enclosed much of the system and was designed to eliminate magnetic fields, suppress extremely low frequency (ELF), and stray noise, such as 60 Hz (Fig. 2). The initial calibration of the system did not apply when the instrumental configuration, the exposure chamber or the sample type were altered; therefore, each change required recalibration. Power delivered was determined at 11 frequencies by measuring incident and reflected power levels.

Fluorescent molecules, at low concentrations produce spectra which may be altered, when the macromolecules about them undergo physical or chemical changes. Such molecules incorporated into the specimen were used to determine potential and phase changes within the membrane.

A voltage clamp, designed and built in our laboratory, enables sensitive control and stabilization of resting potential. It is a valuable tool in calibrating resting potential sensitive membrane probes. This tool is particularly useful in cells which cannot be impaled with microelectrodes. It provides another method of monitoring the potential of such cells or of imposing a membrane potential on a cell.

Biological System

To determine the effects of millimeter waves on resting (Em) and MEPPs the rat flexor digitorum brevis (RFDB) muscle was the model system selected. It was particularly well suited to these studies because of its reported sensitivity to magnetic fields. In addition, its short fibers assure that any intracellular penetration will be close to the nerve-muscle end plate. The nerve and muscle are electrotonically decoupled. In reality, we were attempting to determine the field effects on the presynaptic fiber by sensing the postsynaptic fiber. Tissue culture studies also were carried out on dispersed RFDB fibers, L_6 muscle cells from the American Type Culture Collection (ATCC) as well as fetal and adult cerebellum and spinal cord.

EXPERIMENTS

Experimental and sham specimens placed in sample chambers on a sapphire base apposed to a microwave horn (Fig. 4), were penetrated by microelectrodes to record resting and MEPPs (Figs. 6,7). MEPPs collected in real time were recorded and analyzed for frequency, rise, and decay time, duration, and amplitude. Only those resting potentials above 50 mV and MEPPs with an amplitude above 0.4 mV were

considered acceptable. Muscles were perfused with medium at a rate of 25 ml/hr. The resistance of the electrode was about 20 MΩ. MEPPs were recorded before, during, and after treatment. The various parameters measured, were compared and subjected to an analysis of variance (ANOVA). Baseline studies were carried out with eserine and tetrodotoxin to determine their ability to enhance and stabilize MEPPs.

Resting potentials of cultured L_6 cells (Fig. 6) and of dispersed RFDB muscle cells were determined. The recording of MEPPs in cultured cells requires the production of isolated neuro-muscular junctions. To achieve this, overlay implants of fetal rat spinal cord and cerebellar tissues were grown on a layer of mature, dissociated discrete muscle fibers.

Numerous fluorescent dyes (Fig. 8) were examined to determine optimal staining conditions and to ascertain whether dyes were incorporated into the specimens. Dyes which adversely affected cell behavior or provided inadequate emission spectra were rejected. Of the several dyes deemed suitable, merocyanine 540 was selected to determine potential variations, as it provided the greatest fractional fluorescent change.

Suspended cells, in matched cuvettes containing low and high K^+ concentration in the culture medium, were equilibrated and the emission spectra recorded. By varying the exogenous K^+ concentration, changes in membrane potential could be followed. Calibration of the fluorescence response (Fig. 9) requires the use of microelectrode measurements following chemically imposed changes in membrane potential. Fluorescence changes were examined during the defined periods of irradiation. The relative standard deviation in an ANOVA was used to statistically determine relative changes in fluorescence.

All preliminary experiments in this study were carried out at 51.72 GHz or 51.81 GHz and 5 mW/cm^2 at 33 ^oC. Specimens were irradiated for 10, 30, or 60 min. As this system is an on-line system, continuous monitoring was observed throughout the study. Once the technology was defined, using a single impalement, attempts were made to collect and record 64 MEPPs each, before, during, and after treatment. The instability of the antivibration table, and other stabilizing devices played havoc with the collection process. After numerous combinations and perturbations, the final series of experiments carried out in this study involved 2 block periods: (1) pretreatment (no irradiation) and treatment (irradiation), or (2) treatment and post treatment (no irradiation). During each 5-min phase, 32 MEPPs were collected. Three repetitions of the treatment levels were compared. Experiments were carried out as a completely randomized block. Differences were analyzed by an ANOVA or a Student's T test.

A-1

RESULTS

The system just described was designed, developed, and used in preliminary experiments to determine whether optical and electrical signals can be examined simultaneously to compare RFR effects on excitable tissues. Problems encountered included: major flaws in the manufacturing design of the oscilloscope, instability of the antivibration equipment, and of the Brown-Fleming pipette puller, the need to redesign and realign components of the spectrofluorimeter to permit the optical fibers to be interfaced with the instrumentation and the sample.

Experiments carried out demonstrated it was feasible to measure changes in resting and MEPPs with electrical and optical signals, independently as well as simultaneously, during microwave exposure (Fig. 10). The recorded resting membrane potential of L_6 cells in culture was -60 mV. This response remained quite stable throughout the experiment. Resting potentials and MEPPs collected before, during, and after exposure to millimeter waves at 51.72 or 51.81 GHz and 5 mW/cm^2 for 5, 10, 30, or 60-min intervals at 33 ^oC did not demonstrate significant differences in any of the parameters measured (Figs. 11-18). The frequency and amplitude of MEPPs differ in different fibers within a single muscle as they are electrically isolated.

Merocyanine 540 can be incorporated into the membranes of in vitro neuromuscular preparations. The fluorescence signal of dye incorporated into the cell is significantly greater than the dye molecules in solution. Optimized positioning of the fiber optics provided the ability to monitor relatively small fluorescent signals at locations remote to the spectrofluorimeter. A stronger excitation source would further increase the intensity of the signal. That variations in the extrinsic environment alter the resting potential is visible in fluorescence spectra.

CONCLUSIONS

The experiments carried out demonstrated that the developed prototype system will enable optical and electrical signals of resting membrane potentials and MEPPs to be examined independently as well as simultaneously. However, as fabricated, the system has a major weakness. The fluorescence signal measured is a composite of signals of all the cells in the field. It would be most desirable for this measurement to be specific for a single cell. This function can be accomplished by upgrading the system.

Comparisons of resting membrane and miniature end-plate potentials collected before, during, and after exposure to continuous or pulsed (1 kHz or 16 Hz) millimeter waves did not demonstrate any statistically significant differences at a 95% confidence level, in any of the parameters measured under the stated experimental conditions.

PRESENTATIONS

1. A System for Determining the Effects of Millimeter Waves on Membrane Potential of Rat Muscle Cells. June 1988.

2. Visualization of Millimeter Effects on Membrane Potentials of Nerve-Muscle Preparations. June 1988.

3. Simultaneous Intracellular and Fluorescence Monitoring of Miniature End-Plate Potential in Rat Neuromuscular Preparations During Millimeter Wave Exposure. June 1988.

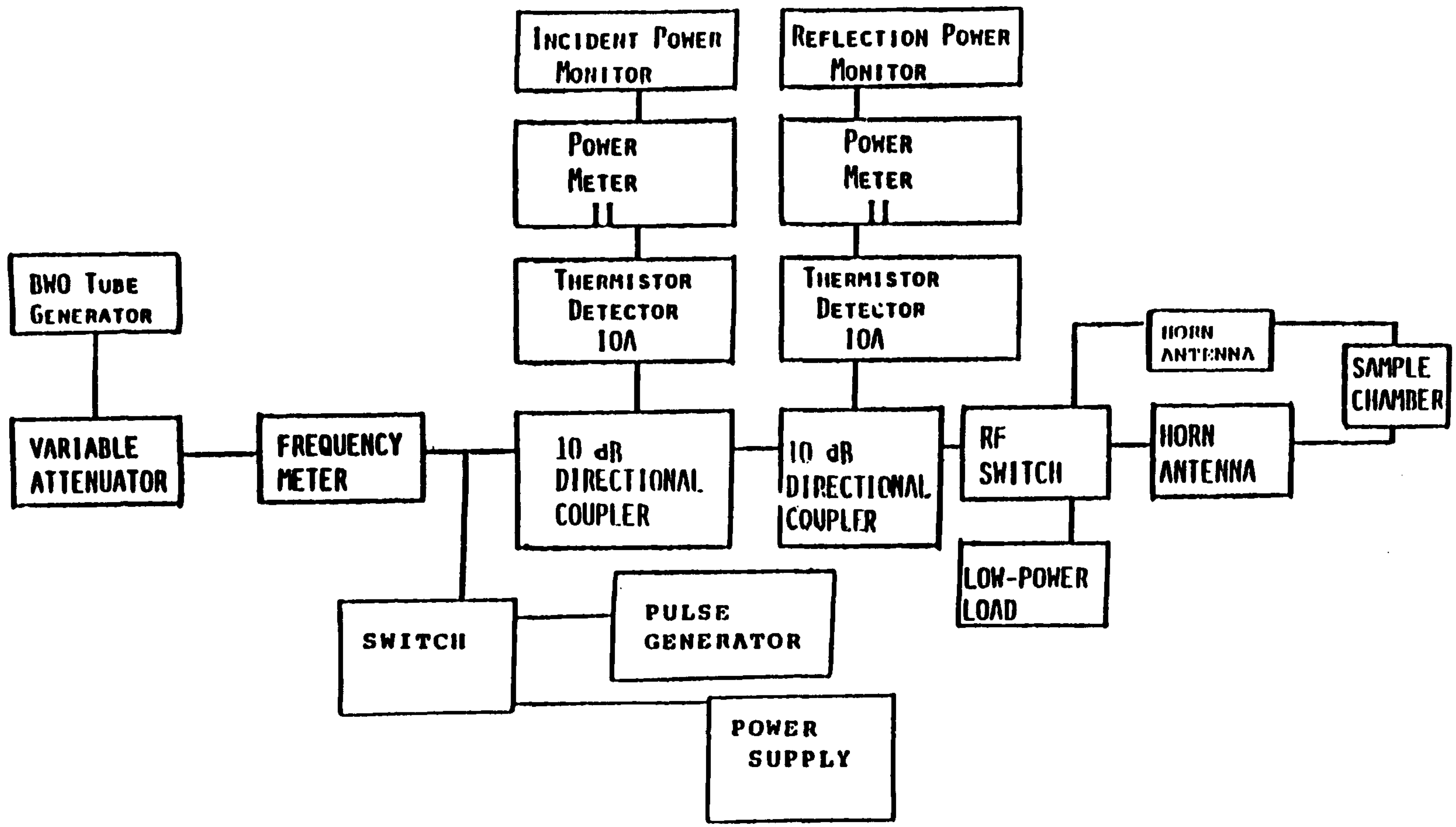


Figure 1. Block diagram of microwave system.

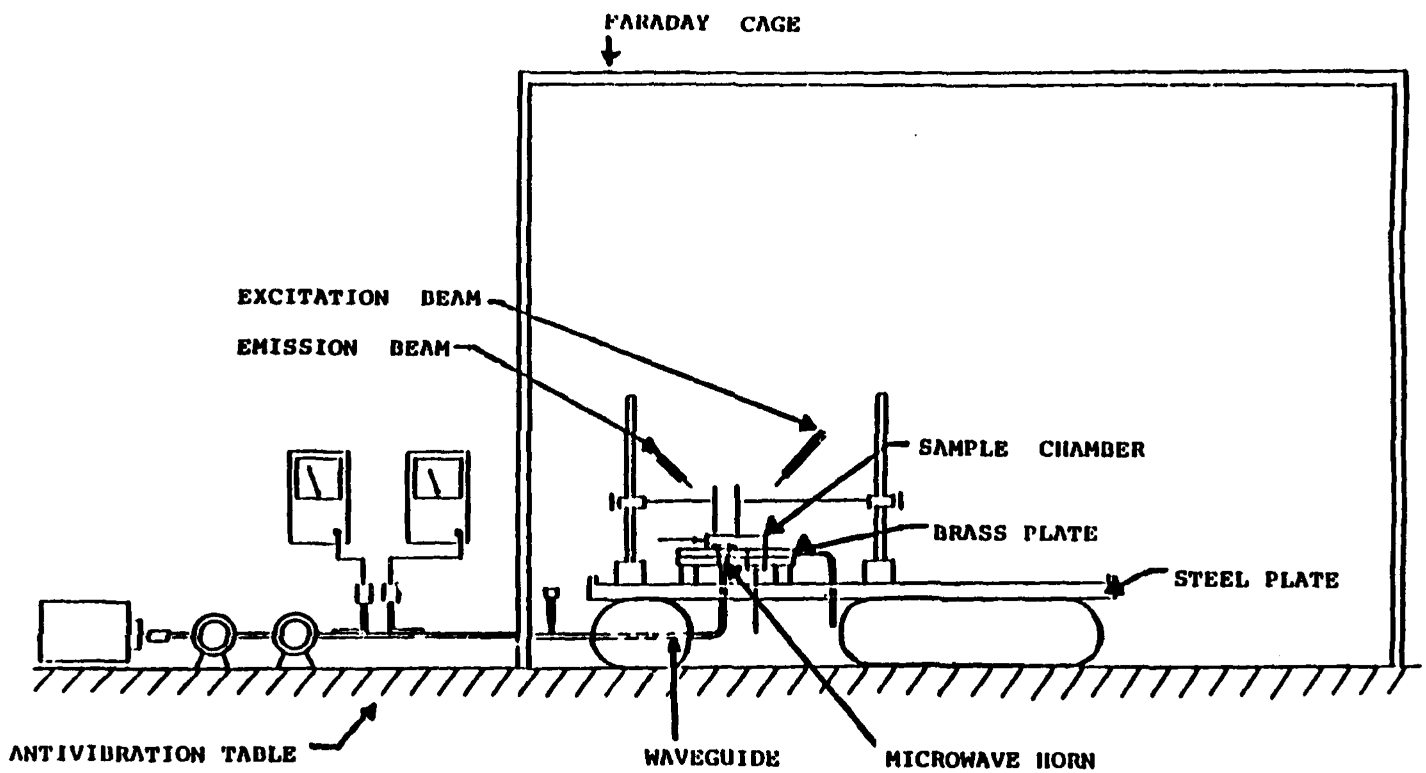


Figure 2. The microwave system interfacing with the sample in the experimental system.

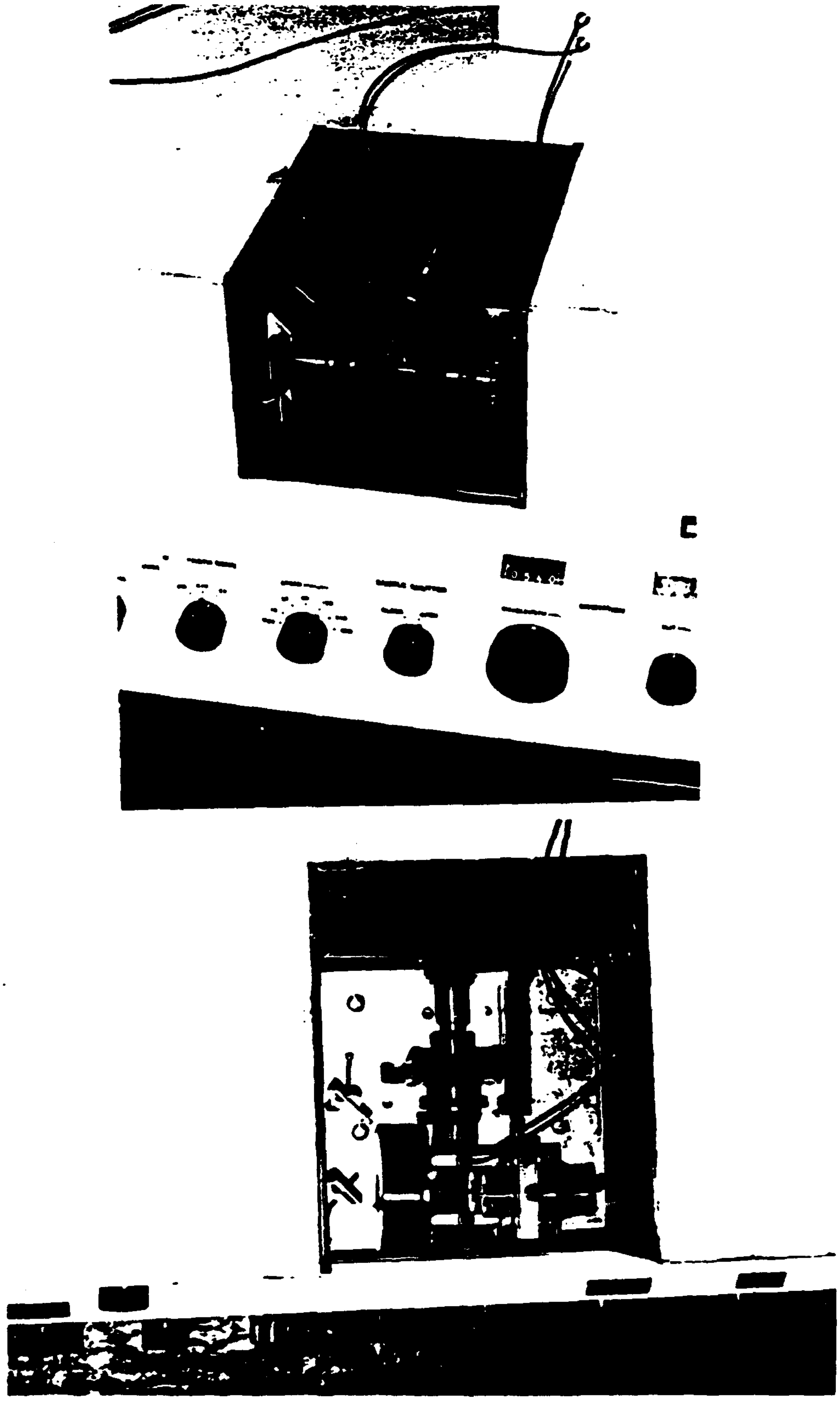


Figure 3. Spectrofluorometer altered to enable optical fibers to interface excitation and emission optics with the specimen.

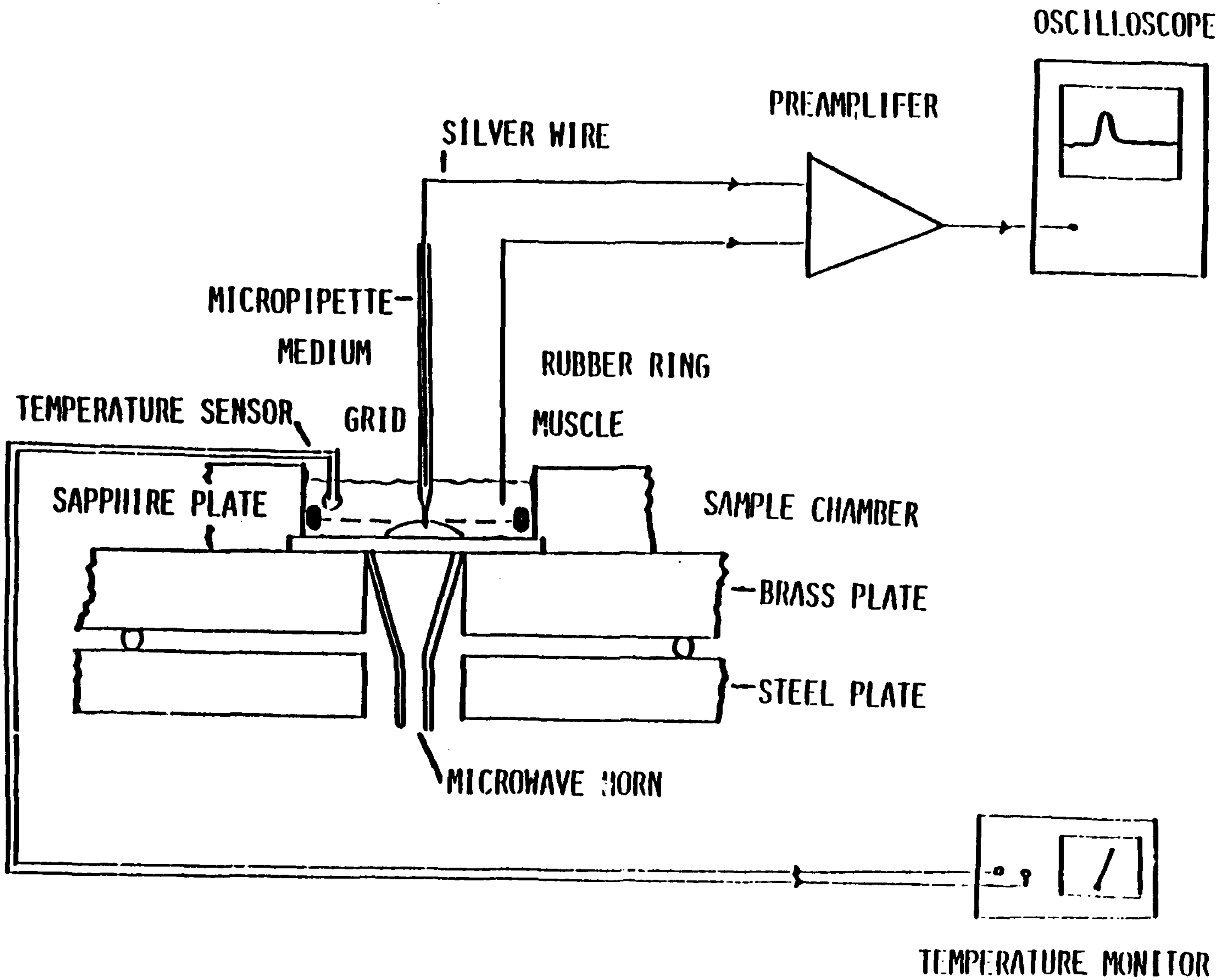


Figure 4. Enlargement of experimental system with microwave horn apposed to impaled sample.

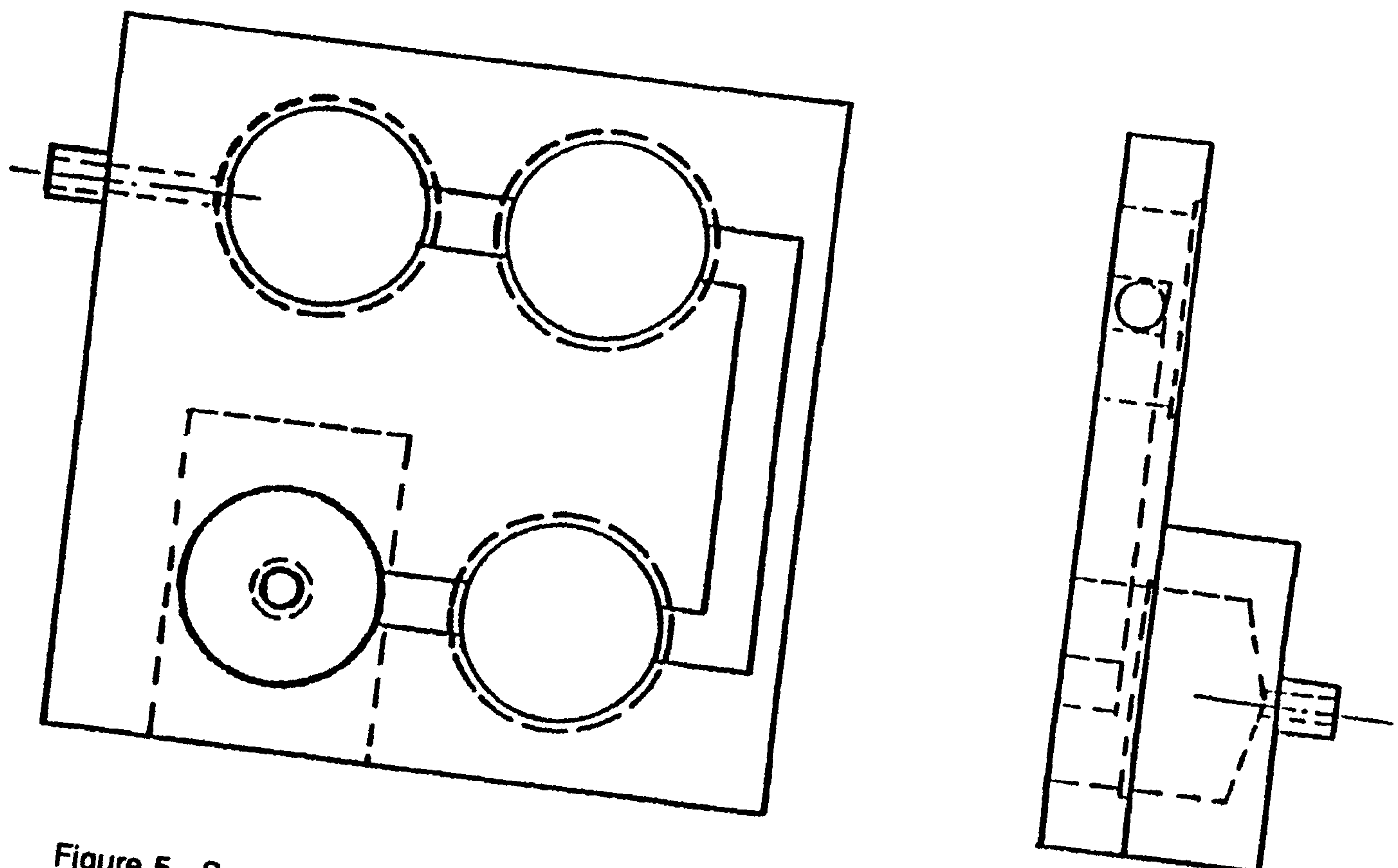


Figure 5. Sample chamber with sapphire plate enables temperature equilibration, control, microwave exposure, and sink for medium.

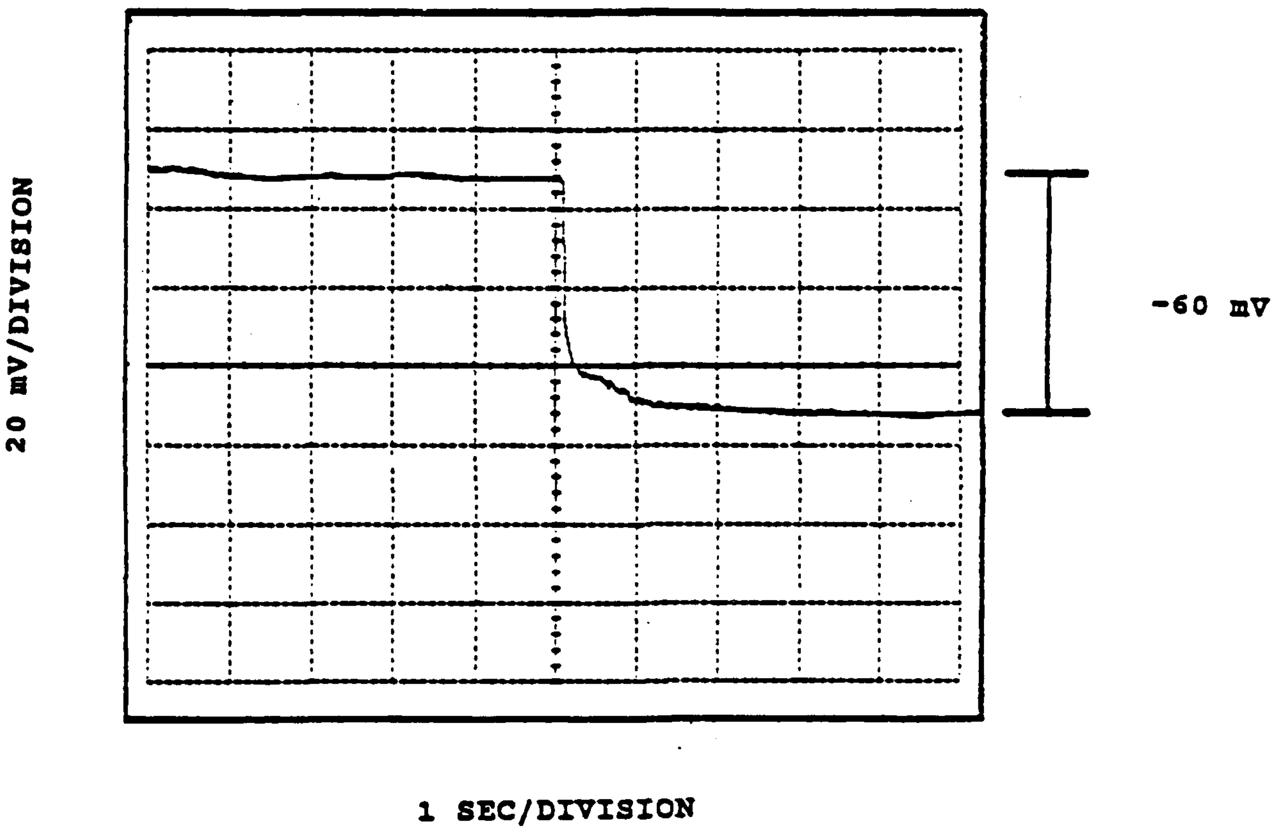


Figure 6. Resting membrane potential of L_6 cell in monolayer culture.

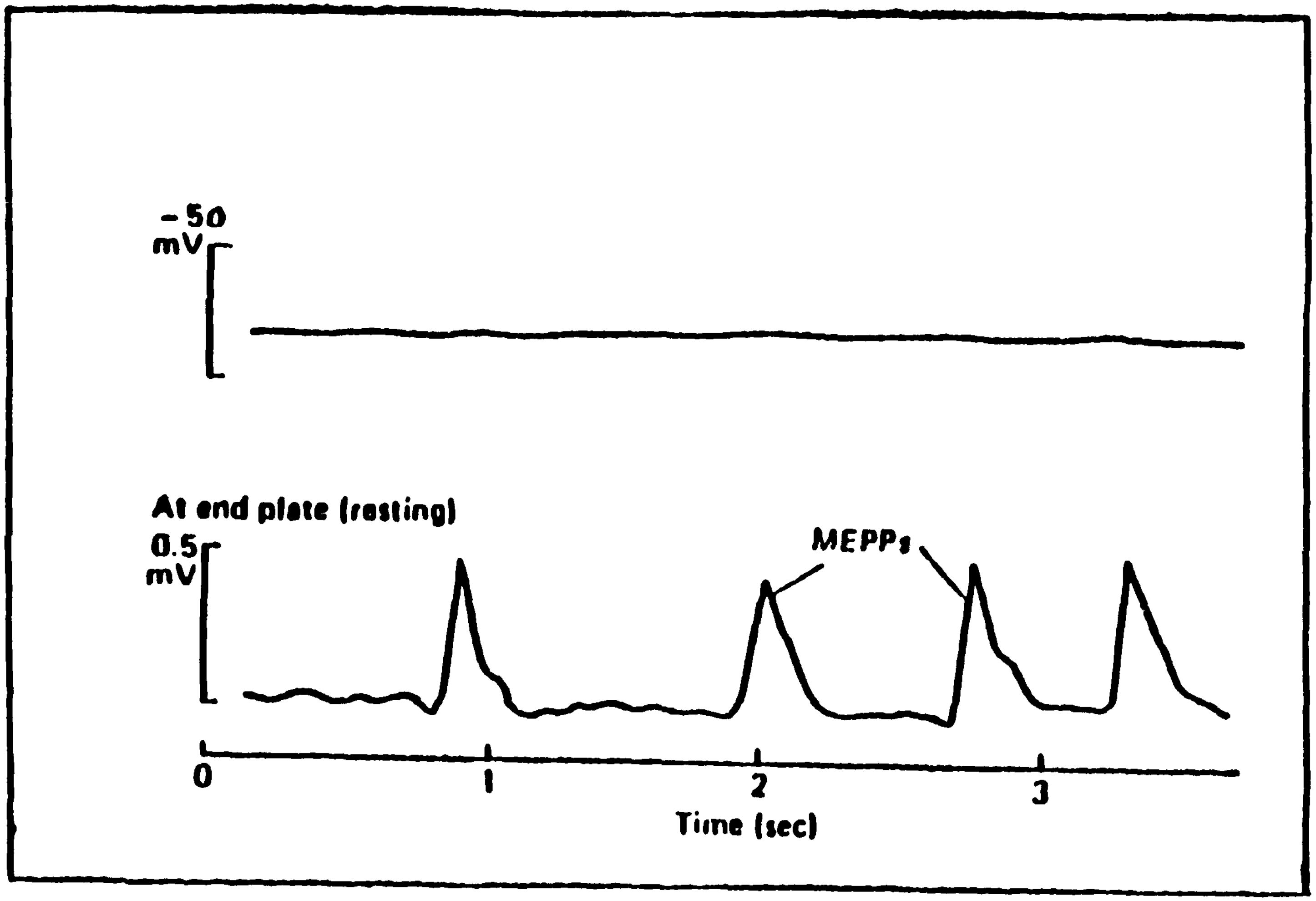
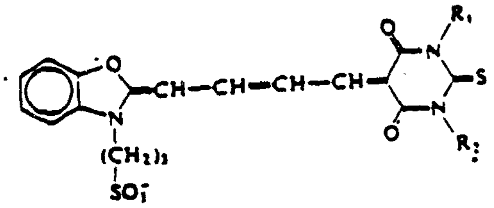
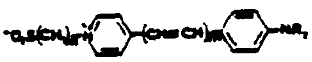


Figure 7. MEPPs of RFDB collected in real time.

MEROCYANINE 540 (M540)

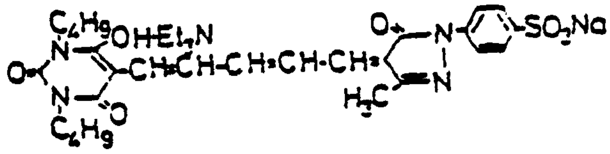


RH160 (FOR BINA HILDESHEIM)

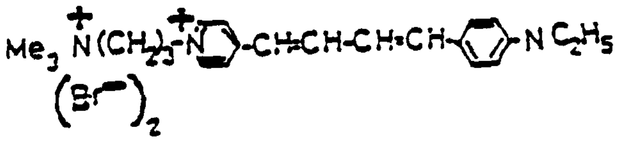


(m=2, n=4, R=butyl)

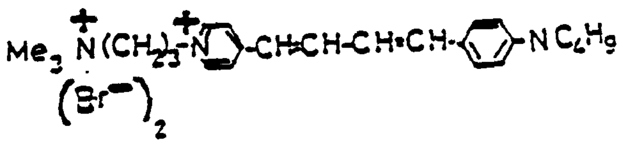
WW781 (= NK 2935)



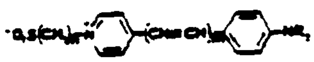
T1111



T1112 (= RH 414)

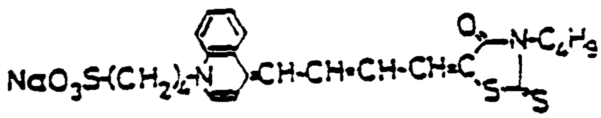


RH 421

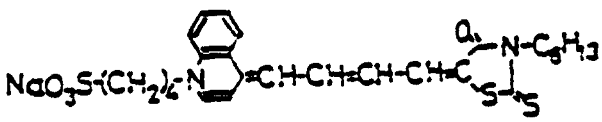


(m=2, n=4, R=pentyl)

NK 2761 (FOR NIPPON KANKOH-SHIKISO KENKYUSHO CO.)



NK 2776



NK 2367

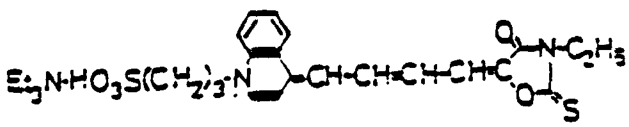


Figure 8. Candidate dyes.

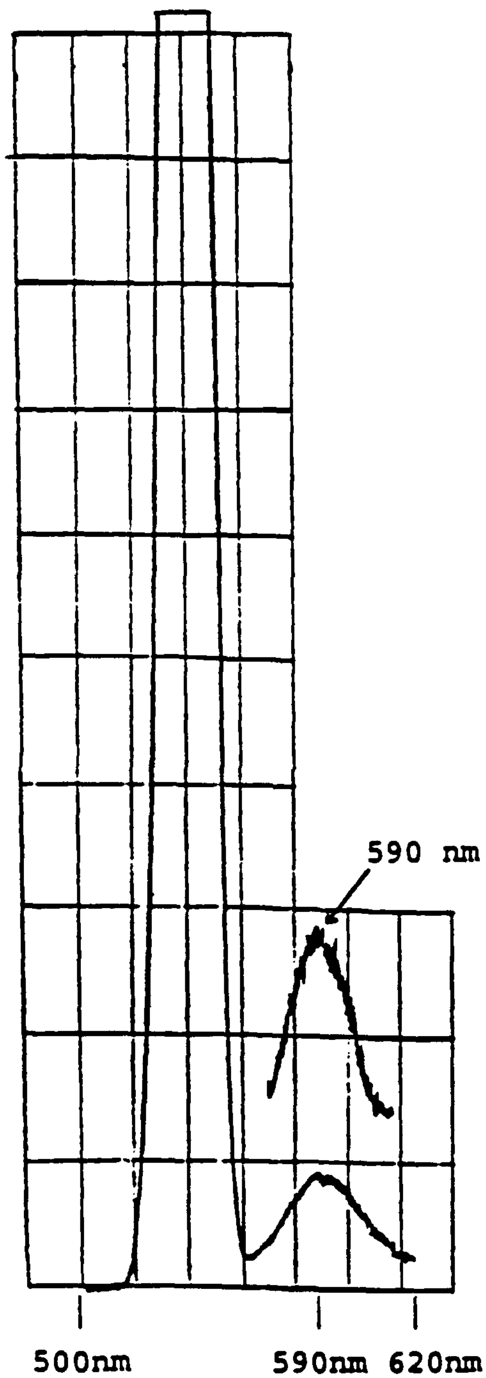


Figure 9. Fluorescence emission spectrum of RFDB membrane with incorporated M540 using fiber optics.

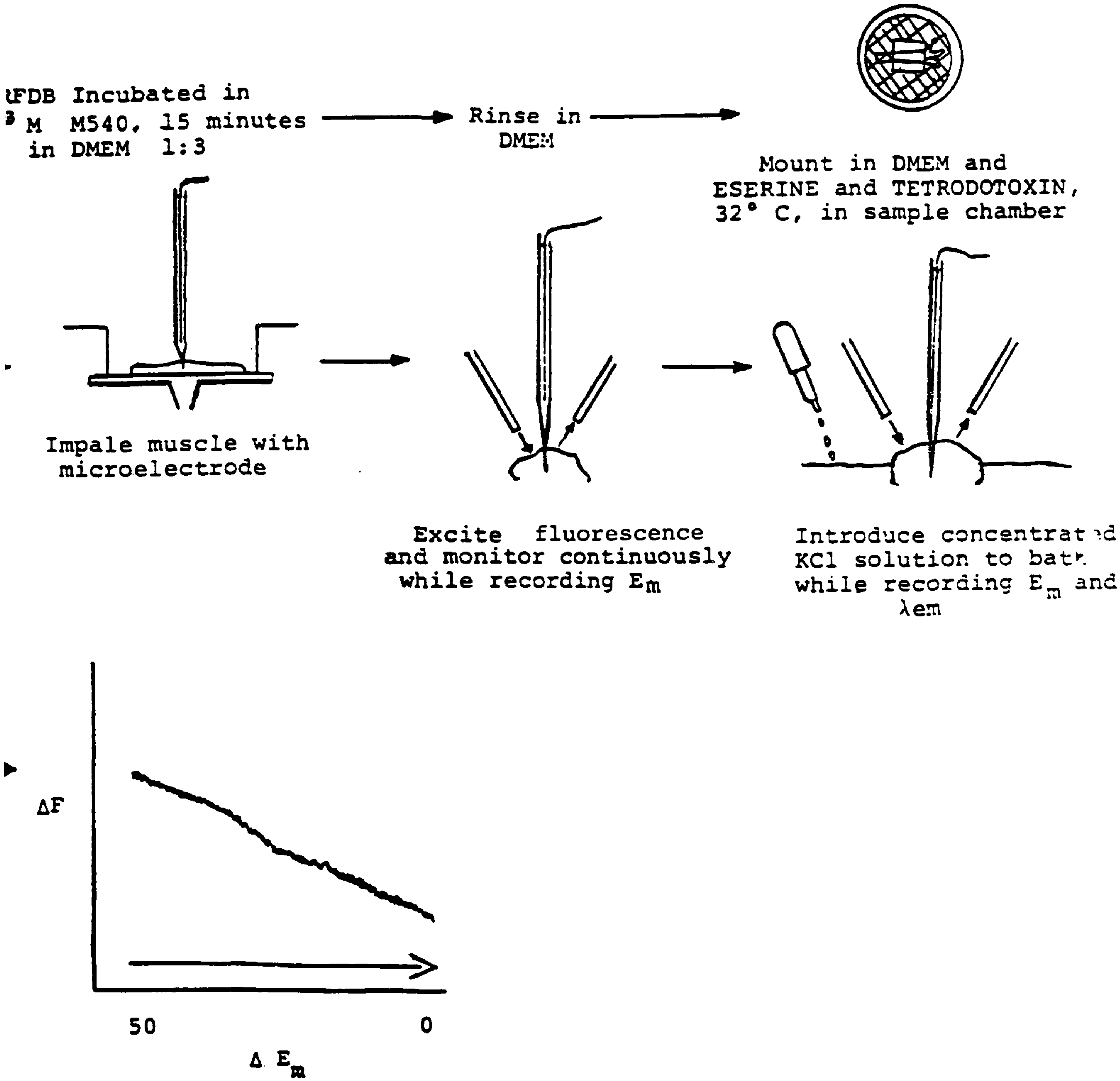
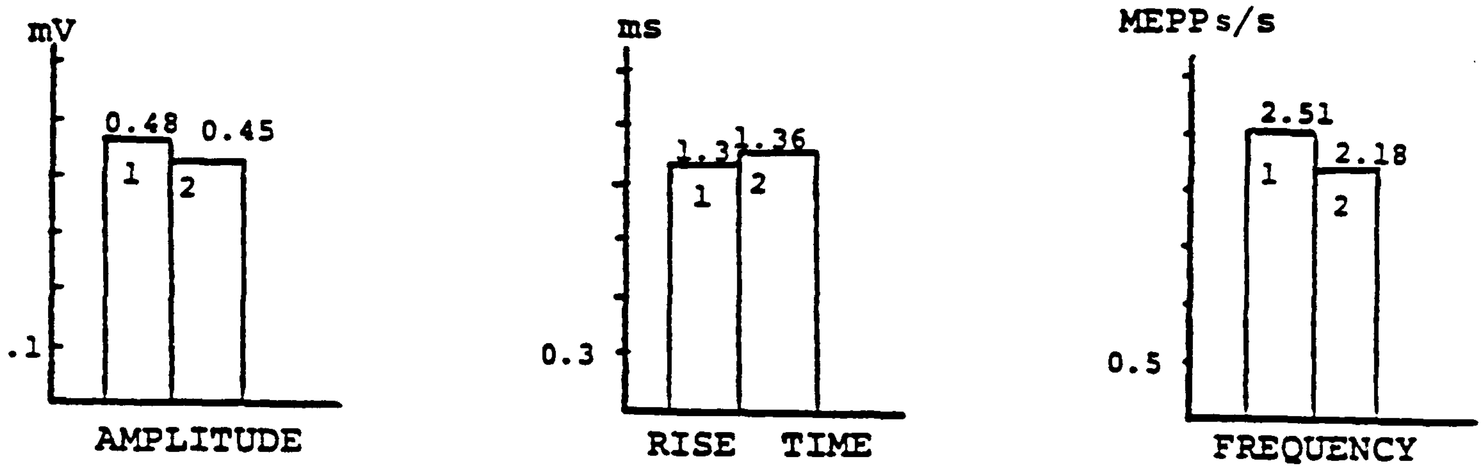


Figure 10. Simultaneous intracellular electrical and optical monitoring of membrane potential in rat muscle.

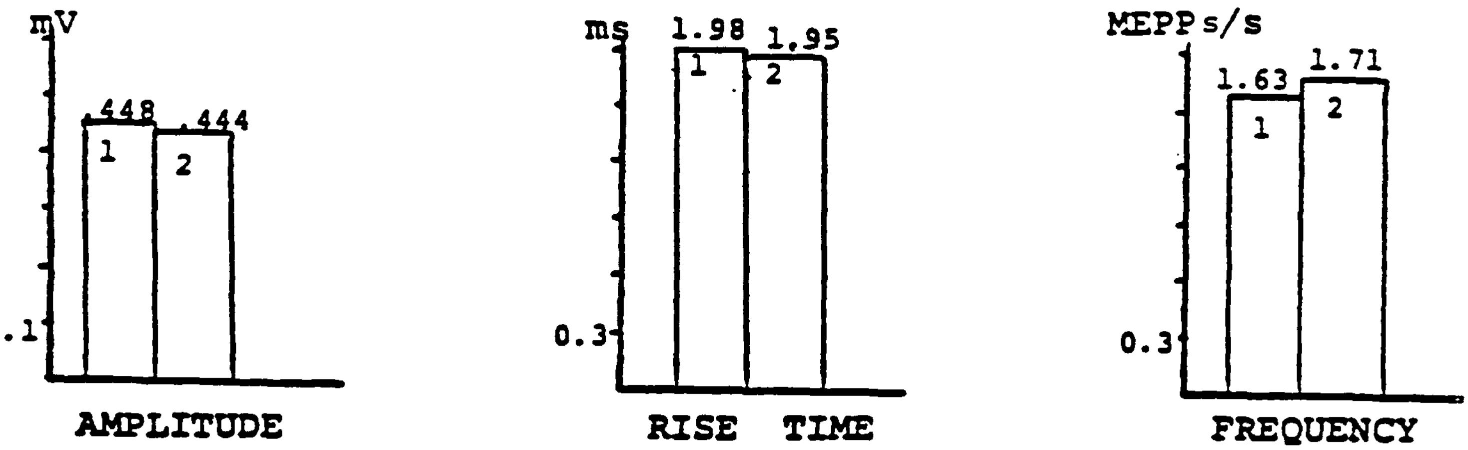
EXPERIMENT 1 : CONTROLS



1. First collection period
2. Second collection period

Em = 38.1
Ēm A ≠ Ēm B

EXPERIMENT 2 : CONTROLS
-With ESERINE and TETRODOTOXIN and vibrations isolated



1. First collection period
2. Second collection period

Ēm = 43.8
Ēm A = Ēm B

Figure 11. Experiment 1. Amplitude, rise time, and frequency of two 5-min collections. No irradiation. Experiment 2. Amplitude, rise time, and frequency of two 5-min collections using eserine and tetrodotoxin.

EXPERIMENT 3:

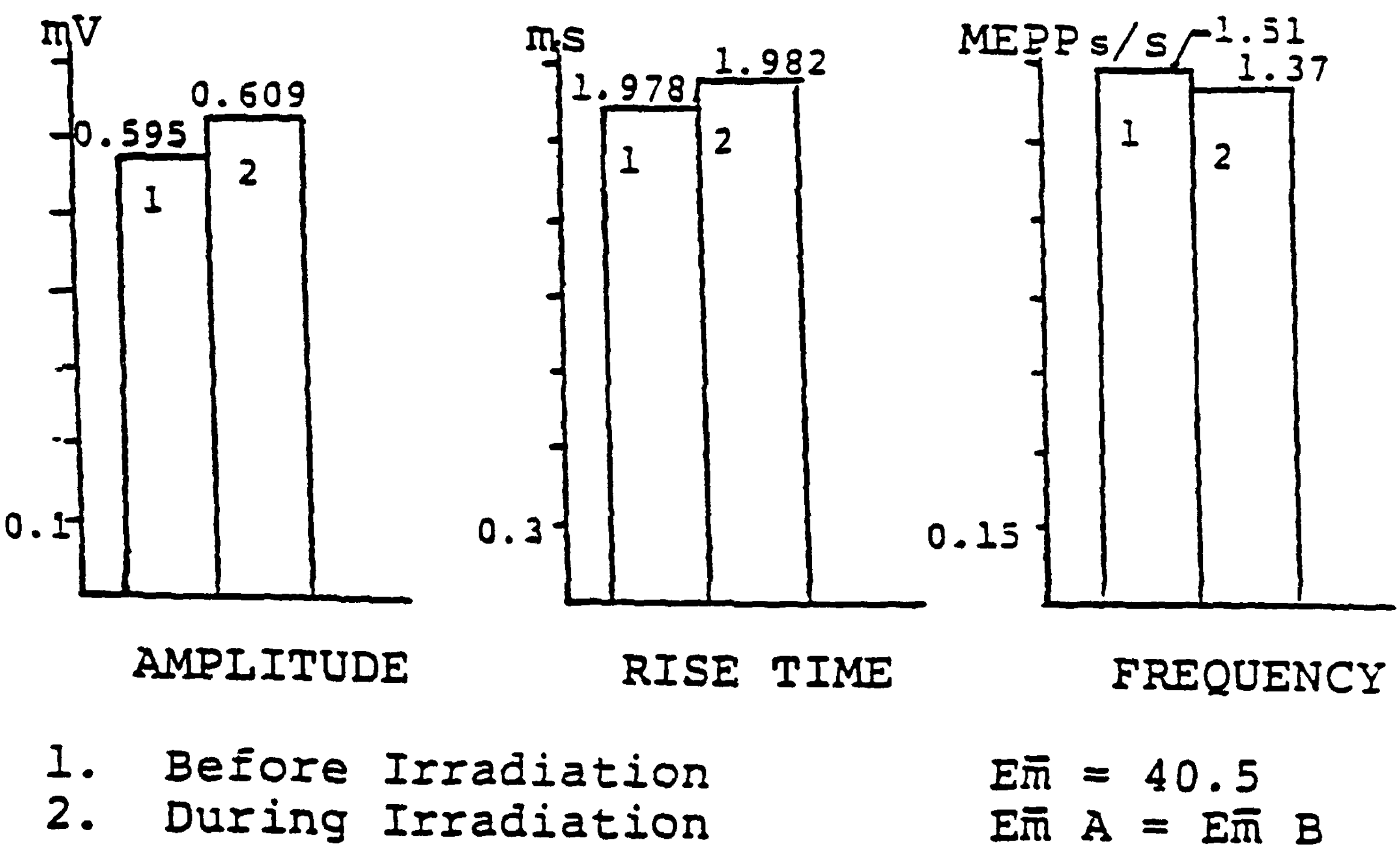
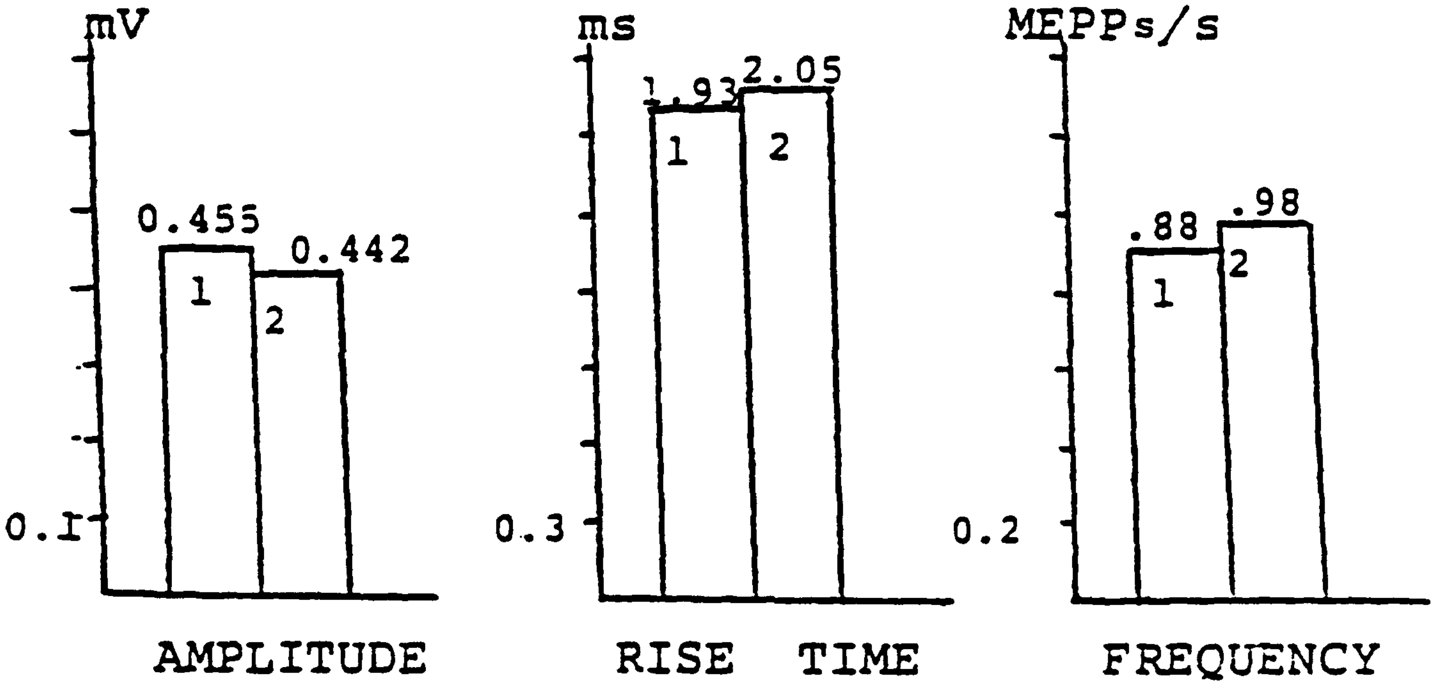


Figure 12. Amplitude, rise time, and frequency in 5-min MEPPs collections.

1. No irradiation
2. Irradiation: CW, 51.81 GHz, 5 mW/cm^2

EXPERIMENT 4:



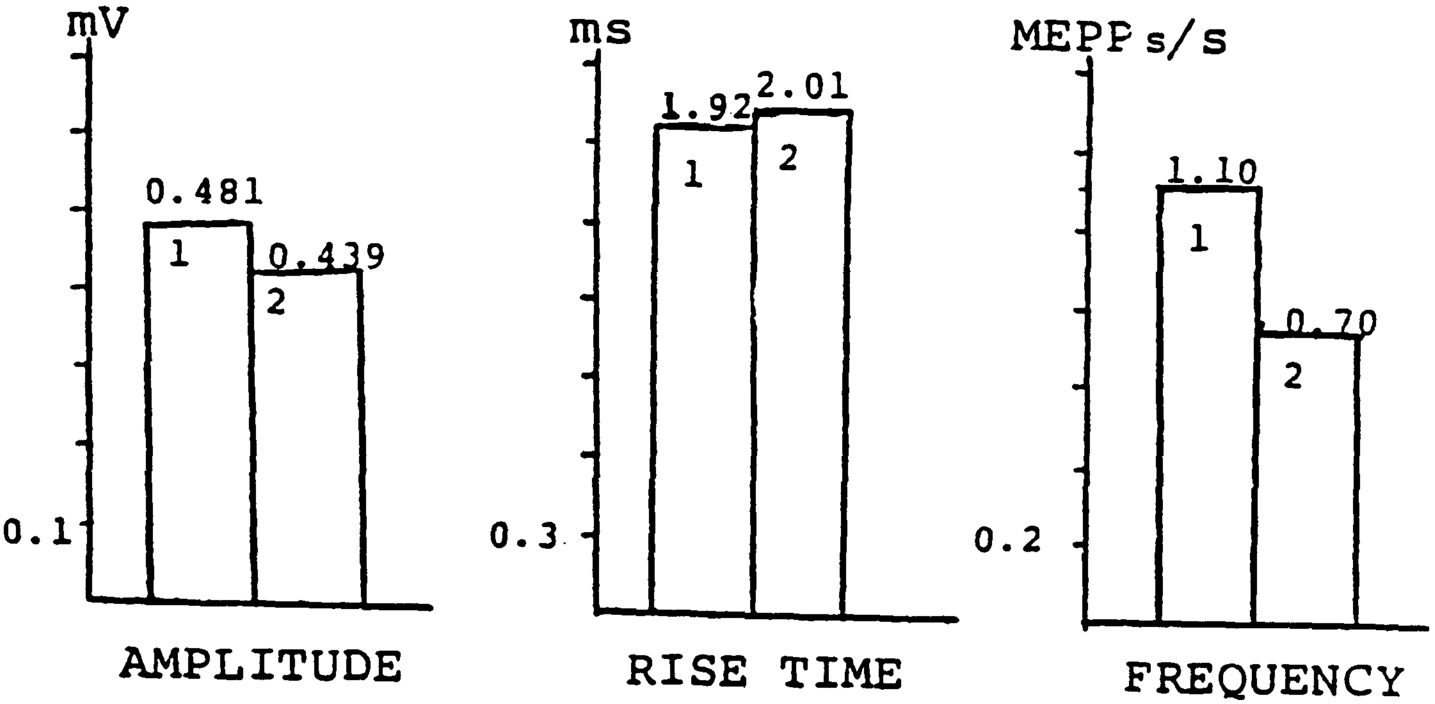
1. Before Irradiation
2. During Irradiation

$E\bar{m} = 41.4$
$E\bar{m}\ A = E\bar{m}\ B$

Figure 13. Amplitude, rise time, frequency in 5-min MEPPs collections.

1. No irradiation
2. Irradiation: Pulsed, 51.81 GHz, 1 kHz, 5 mW/cm^2

EXPERIMENT 5:



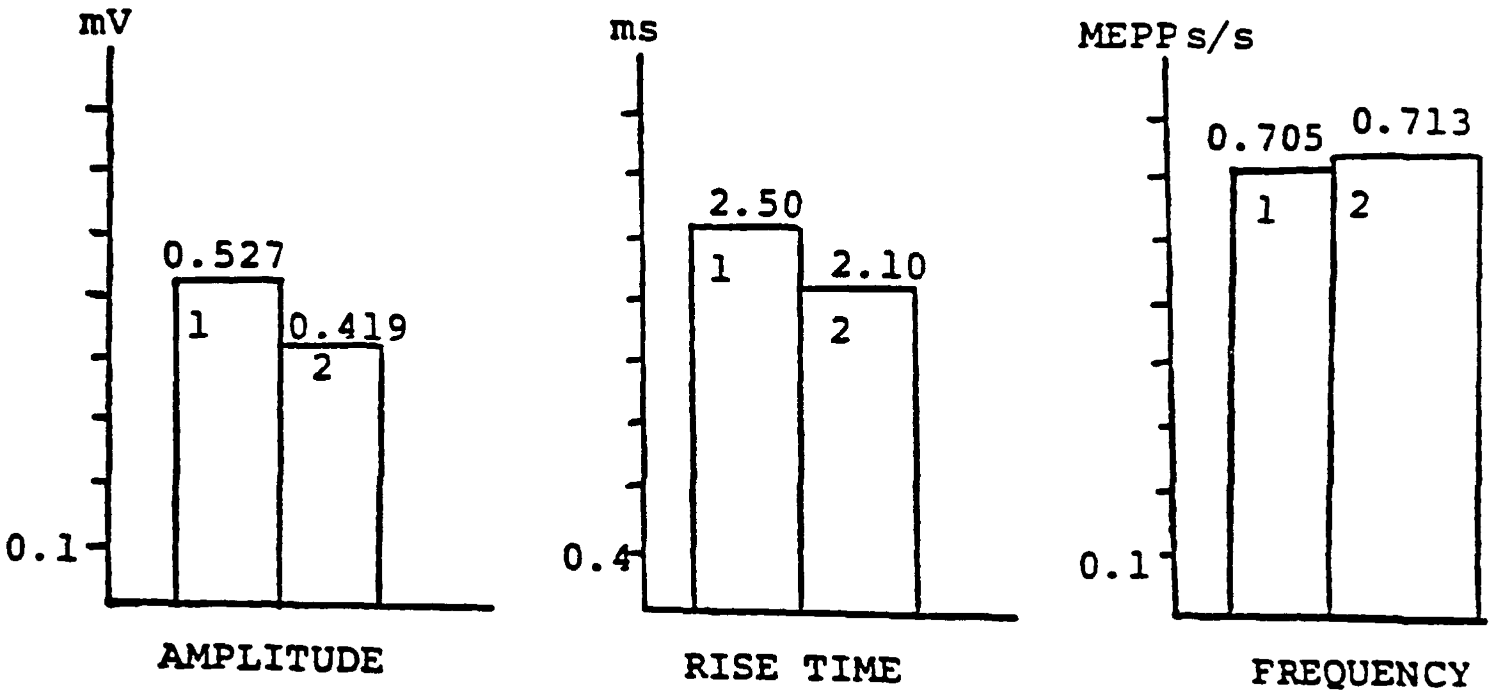
1. During Irradiation
2. After Irradiation

$E\bar{m} = 36.3$
$E\bar{m}\ A = E\bar{m}\ B$

Figure 14. Amplitude, rise time, and frequency in 5-min MEPPs collections.

1. Irradiation: CW, 51.72 GHz, 5 mW/cm^2
2. No irradiation

EXPERIMENT 6:



1. Before Irradiation
2. During Irradiation

$E\bar{m} = 40.0$

$E\bar{m}\ A = E\bar{m}\ B$

Figure 15. Amplitude, rise time, and frequency in 5-min MEPPs collections

1. No irradiation
2. Irradiation: Pulsed, 51.8 GHz, 16 Hz, 5 mW/cm^2

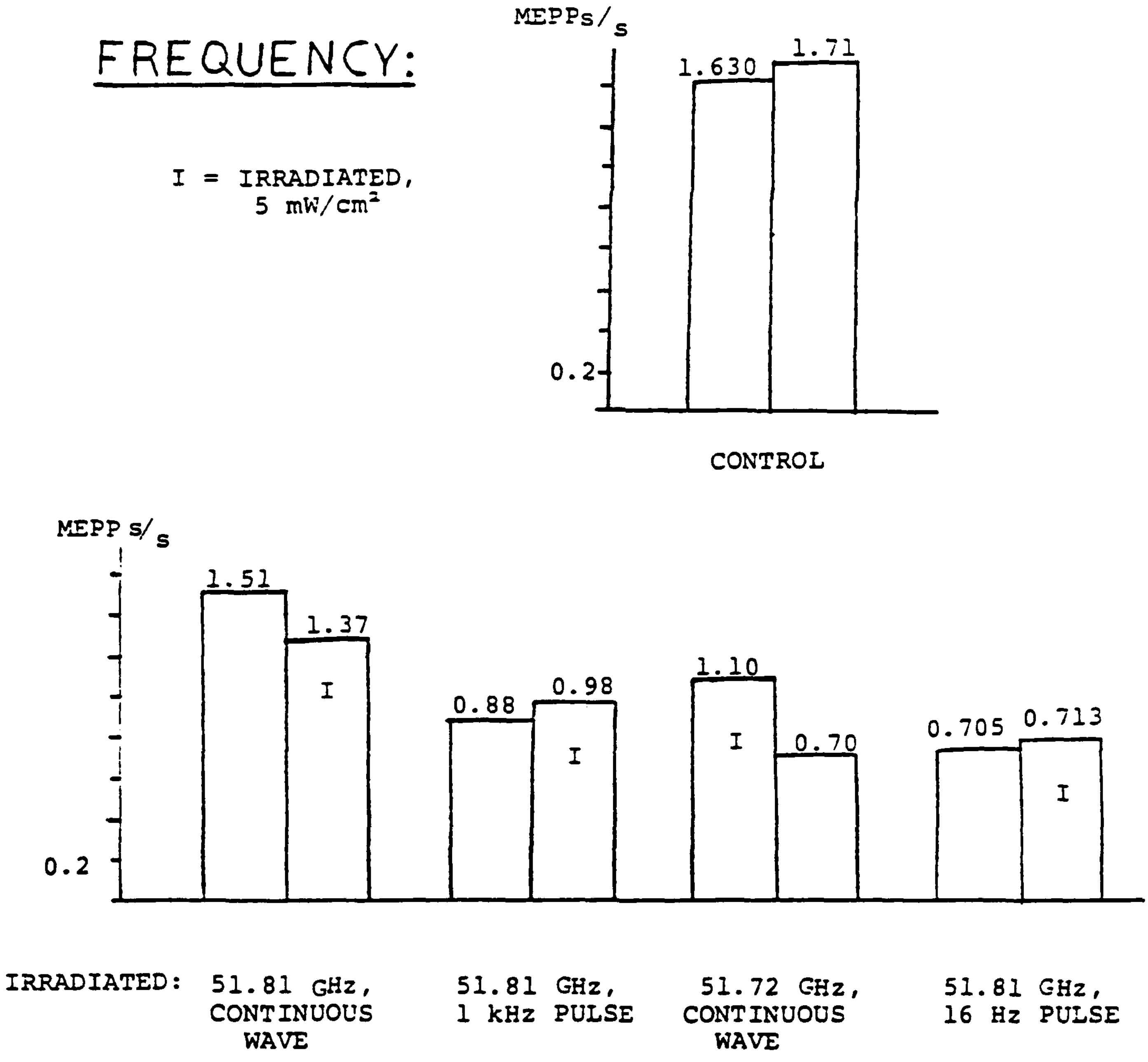


Figure 16. Comparison of frequencies: control; CW 51.81 GHz; 51.81 GHz, pulsed 1 kHz; CW, 51.72 GHz; 51.81 GHz, pulsed 16 Hz.

RISE TIME:

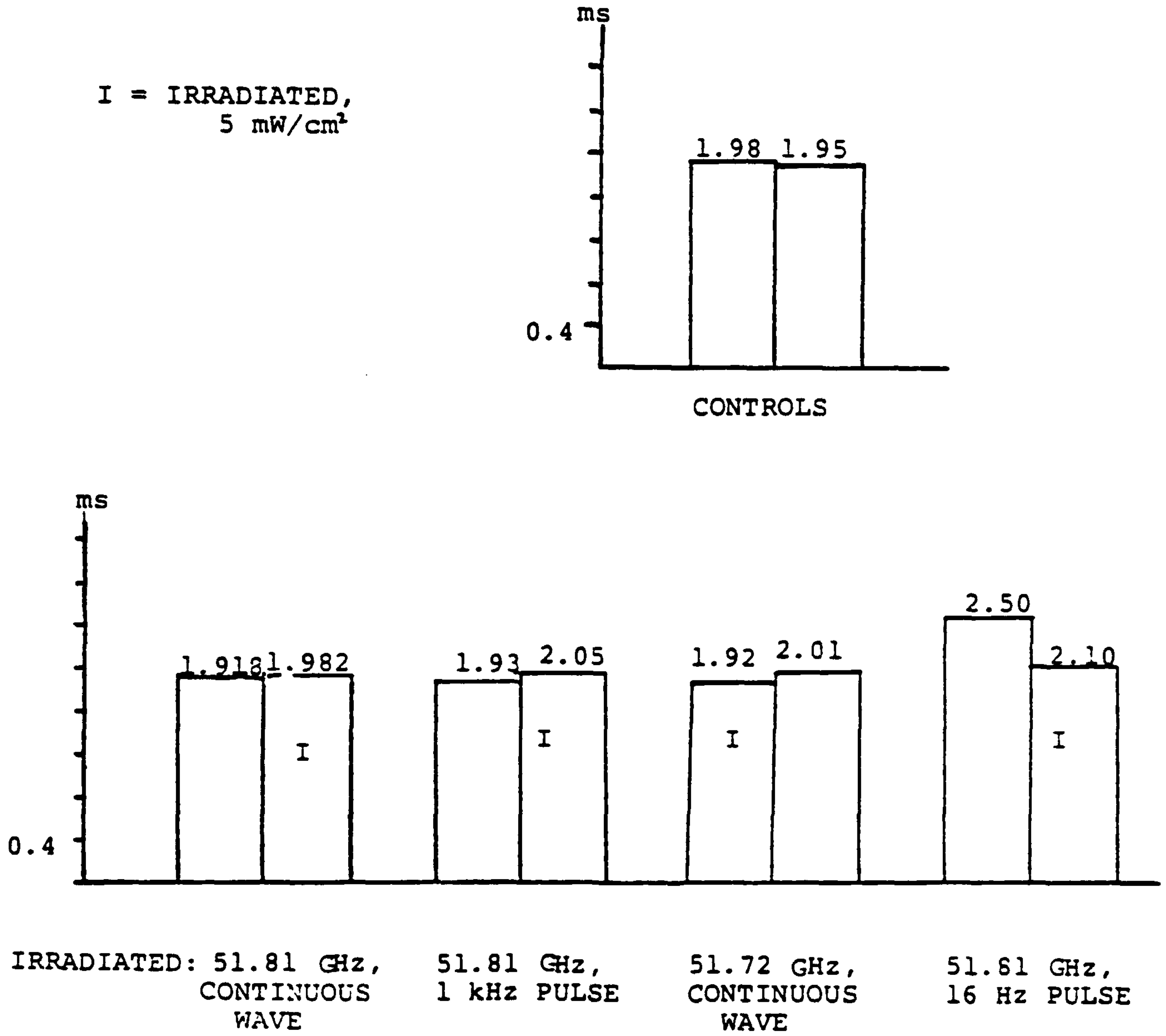


Figure 18. Comparison of rise time: control; CW, 51.81 GHz; 51.81 GHz, pulsed 1 kHz; CW, 51.72 GHz; 51.81 GHz, pulsed 16 Hz.